*TISCIA 34, 19-22*

# GENETIC (RAPD) DIVERSITY IN TWO ARMADILLIDIUM VULGARE POPULATIONS

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*Homor, P., Pfeiffer, I. and Pénzes, Zs. (2003):* Genetic (RAPD) diversity in two *Armadillidium vulgare* populations. — *Tiscia 34, 19-22.*

**Abstract**. In this preliminary study, we investigated the effects of short-term habitat fragmentation on genetic (RAPD) diversity in two *Armadillidium vulgare* populations. Overall, the gene diversity seemed to be moderate. Genetic structure was analyzed at two levels corresponding to two spatial scales: within and between populations. Using different approaches, the overall FST=0.2 suggested genetic differentiation between the populations. The genetic structure of *A. vulgare* populations appears to have been affected by short-term habitat fragmentation.

*Key words: habitat fragmentation, isolation, migration.*

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## Introduction

Fragmentation of natural ecosystems is generally seen to be one of the most important threats to biodiversity (Saunders *et al.* 1991, Miller *et al.* 1995). Fragmentation may occur when human activities, such as agricultural development replace large proportions of the natural ecosystem with a greatly modified matrix, within which small remnants remain. At population level, habitat fragmentation results in decreasing level of gene flow. The reduction of the population size opens the way toward the higher level inbreeding and raising the unwanted importance of genetic drift (Hartl and Clark 1997). Nevertheless, genetic processes of fragmented populations are known to be complex (e.g. McCauley 1991, Hedrick and Gilpin 1997).

The polymorphism revealed by genetic markers provides appropriate resolution for studying population structure (e.g. Sunnucks 2000). In this study, we used randomly amplified polymorphic DNA (RAPD) markers (Welsh and McClelland 1990, Williams *et al.* 1990). Partitioning RAPD variability into different levels of hierarchy, such as within and between populations is often used and considered to be a useful tool for population level studies (e.g. Huff *et al.* 1993, Liao and Hsiao 1998, Vucetich *et al.* 2001).

The aim of this preliminary study was to see whether genetic differentiation could be demonstrated for a common species of which has restricted ability of migration: 150 years of possible isolation and the distance of a few kilometers over a cultivated area can be enough to result in significant differentiation? We investigated the populations of *Armadillidium vulgare* Latreille 1804 (Crustacea, Isopoda, Oniscidae) as it was known to be a common species here. When genetic differentiation is obtained, it supports the hypothesis of isolation. But further detailed studies are needed for a clear test (see discussion).

## Materials and methods

### *Sampling*

Two grassland fragments located in the vicinity of the village Ásotthalom were selected for sampling. These sites are separated by cultivated area (about 5 kilometers). Cultivation is generally dated to be at least 150 years old. The selected species, *A. vulgare* can be characterized by limited capability of migration and sensitivity for

insecticides (Paoletti and Hassal 1999). Although the size of the fragments differs considerably, it was ignored in the analysis. *A. vulgare* populations of the two fragments are denoted by Pop1 and Pop2, where Pop2 is larger.

Individuals were collected in 2002 using pitfall traps. 12 – 36 traps arranged in 1 – 3 regular grid worked for two weeks in the three sampling occasions in both sites. 15 individuals from Pop1 and 30 from Pop2 were used for DNA extraction. The collected individuals were stored in SB puffer (25 mM NaCl, 10 mM Tris-HCl pH 8.2, 1 mM EDTA) at –20 oC, before the preparation. Each individual was handled separately.

*DNA extraction and PCR amplification*

DNA extraction was performed form the internal organs with the exception of the digestive tract using the GeneElute Mammalian Genomic Kit (Sigma). We followed the instructions of the manufacturer. 20 decanucleotide primers (Carl Roth GmbH) were screened for the suitability for RAPD amplification. Three of them (R2: 5'-TGCCGAGCTG-3', R11: 5'-CAATCGCCGT-3', R12: 5'-TCGGCGATAG-3') with the best amplification results were used in the following study. Reproducibility was tested only for the R2 primer. Altogether, 20 polymorphic bands were obtained for the statistical analysis (Table 1).

The PCR reaction was performed in a total volume 20 µl. The reaction mixture contained 2 µl 10x Taq-puffer, 2 µl 2.5 mM MgCl2, 2-3U Taq-polimerase (ZENON Biotech), 0.4-0.4 µl from each dNTP (10 mM, Sigma), 11.2 µl Millipore distilled water, 2 µl primer (10 pmol/µl), and 1 µl template DNA. PTC-100TM Programmable Thermal Controller (MJ Research Inc, USA) was used with the following temperature profile: 94 oC for 2 min; 35 cycles of 94 oC for 1 min, 35 oC for 1 min, 72 oC for 1.5 min.

Amplification products were separated by 1.5% agarose gel electrophoresis (70 mV, 2.5 h) and visualized by ethidium bromide staining. Lambda pUC mix (MBI Fermentas) was used as a molecular weight marker. After digitalization, the banding pattern was scored by eye. The presence of the selected bands was recorded (UVP, Biodoc-ItTM System).

*Statistical analysis*

Clearly, one individual should be enough for many different PCR assay. Because of technical troubles, one individual was analyzed by 1-3 primers. In this manner, statistical evaluation was performed separately on the banding pattern of the same primer. This might have some consequences besides the fact that the analysis was less effective. First, statistical power of discrimination may decrease by combining the results of the tests. Second, results obtained for the different primers are not independent as sometimes the same individual is used in different assays. Ignoring this fact may increase the magnitude of the standard error resulting in false negative decision. Nevertheless, these precautionary facts do not ignore our results as selected methods are very robust (randomization tests) and significant results are obtained (see below).

Statistical analysis was conducted with the assistance of Arlequin software (Schneider *et al.* 1999) and R (Ihaka and Gentleman 1996). RAPD method results in multilocus banding pattern with dominant marker inheritance. We used two approaches for evaluation: banding pattern is considered as a RAPD haplotype (haplotype-based approach) or a band is considered as a locus with dominant allele present (allele-based approach).

Haplotype-based statistical analysis was performed on the full data set. In order to reduce bias, allele-based methods were applied on a data set restricted by the Lynch-Milligan (1994) criterion (Table 1). Applying this condition, 14 loci were obtained and dominant allele frequencies were estimated. These were used then for estimating gene diversity (that is the expected heterozygosity under Hardy-Weinberg equilibrium) implementing the formulae of Lynch and Milligan (1994) in R. Averaging over loci, within population gene diversity can also be estimated. Owing to the lack of information about the level of inbreeding, it was assumed to be zero.

To quantify regional and local genetic structure, we examined genetic variation within and between populations using analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) and fixation indices. First, AMOVA was performed on RAPD haplotypes using Arlequin. The distance between two samples was given by the number of non-shared markers (Excoffier *et al.* 1992). Between populations variance component was converted to FST and tested using 5040 random permutations. Second, the differentiation of the two populations was tested by the Raymound and Rousset method (1995) implemented in Arlequin. It is based on the null hypothesis of panmixia and gives the probability of non-differentiation. It is a nonparametric method, a Monte Carlo approximation of the extended Fischer's exact test. We used 5000 Markov chain steps during the calculations. Third, fixation indices (FST) were calculated in two different ways. FST was given from the gene diversity according to

Table 1. Summary of the sampling and within populations statistics. N stands for the sample size. Shared haplotype means the number of haplotypes can be found in both populations and shared-N is the number of individuals characterized by the shared haplotype in the given population. AMOVA shows the within population variance component given also in the percent of total variance. LM notes the number of loci satisfying the Lynch-Milligan criterion used in the diversity calculations. Variance gives the sampling variance of gene diversity.

| Populations | | N | Haplotype (shared, N) | AMOVA (%) | Locus (LM) | Gene diversity (variance) |
|---|---|---|---|---|---|---|
| R2 | Total | 37 | 19 (1) | 1.351 (72.42) | 7 (4) | 0.30 (0.018) |
| | Pop1 | 10 | 8 (N=1) | | | 0.17 (0.002) |
| | Pop2 | 27 | 12 (N=1) | | | 0.44 (0.002) |
| R11 | Total | 27 | 16 (1) | 1.145 (70.21) | 7 (5) | 0.28 (0.008) |
| | Pop1 | 6 | 5 (N=1) | | | 0.20 (0.001) |
| | Pop2 | 21 | 12 (N=1) | | | 0.37 (0.007) |
| R12 | Total | 40 | 13 (3) | 0.793 (98.56) | 6 (5) | 0.17 (0.002) |
| | Pop1 | 30 | 11 (N=16) | | | 0.21 (0.003) |
| | Pop2 | 10 | 5 (N=7) | | | 0.12 (0.001) |
| Combined | Total | 114 | 48 (5) | - | 20 (14) | 0.25 (0.003) |
| | Pop1 | 46 | 24 (N=18) | | | 0.19 (0.001) |
| | Pop2 | 58 | 29 (N=9) | | | 0.30 (0.003) |

Table 2. Genetic differentiation measured by the two interpretations of FST, the between population variance and gene diversity. Allele-based results are not tested. Non-diff. stands for the p value of the Raymond-Rousset test. The estimation of the error on the calculation of p values is also given. Overall sample X2 is tested on $\chi 2$ (6 degrees of freedom), used for overall testing of differentiation (Fischer's method, Sokal and Rohlf 1995). See Table 1 for simple sizes and within group diversity and variances. NS: $p > 0.05$; ***: $p<0.001$; *: $0.01<p<0.05$;

| Primer | AMOVA (%) | FST (haplotye) | Non-diff. p (error of p) | Gene diversity | FST (allelic) |
|---|---|---|---|---|---|
| R2 | 0.515 (27.58) | 0.276*** | 0.000*** (0) | 0.130 | 0.299 |
| R11 | 0.486 (29.79) | 0.298*** | 0.0259* (0.0068) | 0.054 | 0.159 |
| R12 | 0.012 (1.44) | 0.0144NS | 0.306 NS (0.0184) | 0.004 | 0.022 |
| Combined | - | X2=39.629*** | X2=28.096*** | 0.058 | 0.189 |

Lynch and Milligan (1994). On the other hand, FST was derived from the AMOVA calculations (haplotype-based). We considered allele-based estimates only on qualitative manner.

Overall conclusion based on the haplotype approach was obtained using the Bonferroni and Fischer methods with overall significance level of 0.05. Even the classical Bonferroni method is considered to give good results when the number of tests is small (three in our case) (Rice 1995). Fischer's technique combines the probabilities of different tests of significance (Sokal and Rohlf 1995).

## Result

Gene diversity of the two populations differed considerably, showing larger value for the larger population (Table 1). However, this difference should be interpreted carefully according to the unbalanced samples sizes. Nevertheless, the relatively high number of haplotypes suggests high level of diversity in both populations (not tested). For example, in the case of R2 primer in Pop1 10 individuals carry 8 unique RAPD haplotypes (Table 1).

The number of shared haplotypes between populations suggests also high level of genetic differentiation. One individual was found in both populations what shared the same haplotype in the case of R2 primer (Table 1). However, this trend varied. AMOVA carried out on the full data set showed high level of differentiation, with high values of FST for primers R1 and R2 (0.276 and 0.298, respectively, Table 2). As these were highly significant, the overall test was also significant for both of Bonferroni and Fischer methods (Bonferroni: $p<0.001$ for R2, therefore overall $p<0.003$; for Fischer's method see Table 2.). In case of primer R12, two common shared haplotype detected. This was found in 21 individuals altogether. Using Raymound-Rousset method similar conclusions were obtained. High level of differentiation was found (Table 2). Trends revealed by the allele-based methods were also similar (not tested).

## Discussion

*Armadillidum vulgare* is generally considered to be a good "indicator species" (Paoletti and Hassal 1999) for studying questions of conservation.

Clearly, emphasizing a single species oversimplifies the problem as the level of polymorphism it shows depends on many factors, including biotic ones. Nevertheless, *A. vulgare* seems to be very variable at molecular level. In a world wide study using enzyme polymorphism of *A. vulgare*, Garthwaite *et al.* (1995) reported high level of genetic variability within and between populations, especially in Europe. Similar result was obtained in a smaller spatial scale using genetic markers (Rigoud *et al.* 1999) as it was also suggested by our results.

Further evidences are provided for the huge list of detrimental effect of human activity for natural populations. The Hungarian Great Plain, where our sampling sites are located is not an exception; 150 years of agricultural activity may isolate grassland fragments completely in a short spatial scale.

Finally, the explanation based on isolation of populations is only one possibility. Many background processes can explain differences in genetic composition of populations. Genetic markers are useful for providing high level of polymorphism but (usually) the background processes remain unclear. The effect of migration (the lack of isolation) is to eliminate differences between populations (e.g. Hartl and Clark 1997). But it takes time. Ancestral differences traced back to the foundation (the time of fragmentation) or subsequent historical events (bottle neck) may explain current differences even the lack of complete isolation. Genetic differences can be generated on many different ways. High level of differences can be interpreted as the sign of isolation but migration rate must be also estimated on the direct way (Bossart and Prowell 1998, Steinberg and Jordan 1998, Waser and Strobeck 1998).

## Acknowledgment

During this research, Zs. Pénzes was supported by "Békésy György" grant of Hungarian Ministry of Education.

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